

Project Report

Arizona Iceberg Lettuce Research Council

For period: July 2004 through June 2005

Title: Determination of *Mirafiori Lettuce Virus* and *Lettuce Big Vein Virus* Incidence and Genetic Variability in Big Vein Infected Lettuce.

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INTRODUCTION

Big vein is a viral disease of lettuce (*Lactuca sativa* L.) transmitted by the soil-borne fungus *Olpidium brassicae* (Jagger and Chandler 1934). Symptoms of big vein include chlorosis surrounding the vascular bundles in the leaf and increased stiffness of the leaves that disrupts normal head development, resulting in plants that have a bushy appearance. Reduced frequency of head formation is the primary source of economic damage resulting from big vein disease. Big vein is most prevalent in cool wet soils (Campbell and Grogan 1963, Westerlund *et al.* 1978a, 1978b), and increases with continuous lettuce production without rotation. Consequently, big vein consistently occurs at high levels during spring production in California's coastal growing districts, and during winter production in Arizona.

Effective long-term control of big vein disease is best accomplished through genetic resistance, and is important for sustainable production of quality lettuce. Complete resistance to big vein has only been identified in accessions of *L. virosa* L. (Bos and Huijberts 1990), but this resistance has not been introgressed into lettuce cultivars to date. Among cultivated lettuce, partially resistant cultivars are available that have a reduced frequency of symptomatic plants and/or symptom expression that is delayed until plants reach market maturity (Ryder and Robinson 1995). This type of resistance has greatly improved marketable yields in fields infested with big vein (Ryder 1979). Progress in increasing the level of partial resistance has been slow, primarily because of a lack of information regarding the pathogen, the unknown inheritance of resistance, and the large influence that environmental conditions have on symptom expression.

Although big vein disease has impacted lettuce production for many years, the causal agent, *Mirafiori lettuce big-vein virus* (MLBVV) (genus *Ophiovirus*), formerly known as *Mirafiori lettuce virus*, was only recently identified (Lot et al., 2002). Another virus, *Lettuce big-vein associated virus* (LBVaV) (genus *Varicosavirus*), formerly known as *Lettuce big vein virus*, was previously found associated with big vein disease, but a causative relationship was never confirmed (Huijberts et al. 1990; Vetten et al. 1987). Interestingly, many studies have demonstrated that plants exhibiting big vein symptoms were frequently coinfecting with both viruses, suggesting LBVaV may also contribute in some manner to disease (Roggero et al., 2003; Navarro et al., 2004, 2005).

Understanding the distribution of MLBVV and LBVaV in Arizona and California and the genetic relationships among virus isolates affecting western production is important for

developing control methods suitable for production conditions in the western U.S. Additionally, knowledge of the virus(es) responsible for big vein disease provides an opportunity to develop more effective methods of screening for resistance, and identification of plants not only with reduced symptom expression, but also with reduced virus incidence. Coupling these methods will facilitate more reliable resistance testing than those used previously by lettuce breeders.

MATERIALS AND METHODS

Collection of field samples and classification of symptom severity. Lettuce leaf samples were collected for virus RNA isolation from field sites in the Yuma, Arizona production area and in some instances from the California central-coast production area. Five to nine plants per site were sampled by collecting one complete leaf per plant. Leaves were stored on ice, brought into the lab and classified as healthy, mild, moderate, or severe for big vein symptoms using a disease severity scale as described in Figure 1. Lettuce tissue was sampled (100 mg per sample), lyophilized, and stored at -80°C prior to RNA extraction.

Greenhouse testing of big vein resistance. Greenhouse experiments were performed to compare big vein resistance among *L. sativa* cultivars and *L. virosa*. Experiment 1 used three separate inoculations of the cultivars Great Lakes 65, Pavane, and Margarita with three replications of 12 plants. Experiment 2 used Great Lakes 65, Pavane, and *L. virosa* accession IVT280 in five inoculations of 1 to 3 replications of 12 plants. Each inoculation was conducted by following the protocol of Ryder and Robinson (1995). Seedlings were germinated in a sand-field soil potting mix and grown for three weeks. A suspension of *O. brassicae* zoospores was prepared by macerating the roots of big vein symptomatic plants in water. The seedlings were inoculated by watering the zoospore suspension into the seedling pots twice per inoculation, with inoculation intervals separated by 1 day. Each seedling was subsequently transplanted into an 8 cm pot containing field soil. Plants were grown in a greenhouse maintained at 18°C, and the percentage of symptomatic plants was recorded after 6 to 8 weeks of growth. Tissue was sampled from asymptomatic and symptomatic plants from the third experiment for RNA extraction.

RT-PCR, sequencing, and diversity analysis. Tissue samples were ground in liquid nitrogen, and total RNA extracted using the Qiagen RNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's recommendations. RNA extracts were stored at -80°C. MLBVV and LBVaV coat protein RT-PCR primer pairs were designed from published MLBVV and LBVaV sequences. RNA extracts (as well as positive and negative controls, and reagent blanks) were reverse-transcribed, and cDNA was amplified by polymerase chain reaction (RT-PCR). RT-PCR reactions were electrophoresed on 1% agarose stained with ethidium bromide, and the presence or absence of the target band determined. All samples from which MLBVV or LBVaV RNA did not amplify were re-analyzed using a different primer pair, to rule out the occurrence of false negatives, and ultimately re-tested with molecular probes for LBVaV and MLBVV by nucleic acid hybridization.

Nucleic acid sequence diversity. DNA sequencing was performed on one sample from each field. A portion of the coat protein region of both LBVaV and MLBVV was sequenced for each sample. RT-PCR was performed on these samples using primers with 5'-M13 tails (Table 1). PCR products were purified prior to sequencing with Qiagen kits (QIAquick® PCR Purification Kit, or QIAquick® Gel Extraction Kit; Qiagen, Inc., Valencia, CA). DNA sequencing was also performed on clones of PCR-amplified products for comparison with

sequence generated directly from PCR products. PCR products (one from each of the amplified regions) were cloned using the TOPO TA Cloning® Kit (pCR®II-TOPO®, Invitrogen, Carlsbad, CA) and used to transform *E. coli* (either TOP10 cells, Invitrogen, Carlsbad, CA or NovaBlue cells, EMD Biosciences, Madison, WI) using standard conditions. Plasmids were prepared with the Qiagen Plasmid Mini Kit prior to sequencing.

Sequencing reactions were performed on purified PCR products or plasmids with the USB Cycle Sequencing Kit (USB, Cleveland, OH), using ddNTP termination mixes, and with LI-COR IRDye™-labeled M13 primers (LI-COR Inc., Lincoln, NE). Sequence data were generated using the LI-COR Global Edition IR² System and LI-COR eSeq software (v. 3.0) located at the USDA-ARS in Salinas, CA. Some samples were sequenced by MCLab (South San Francisco, CA).

RESULTS AND DISCUSSION

Virus incidence in symptomatic lettuce

Plants exhibiting big vein symptoms were collected from fields throughout the Yuma growing region in Arizona and tested by RT-PCR for the presence of MLBVV and LBVaV. In all other areas of the world where big vein is found and where attempts have been made to identify these viruses, both have been found together in the majority of plants tested. Only MLBVV is necessary for big vein disease to occur, but the relationship between co-infection with LBVaV and symptom development has not been well characterized. Among plants sampled from the Yuma area, all those exhibiting big vein symptoms, as well as some that had not yet developed symptoms contained MLBVV, as expected. Interestingly, none of the plants from the Yuma area, symptomatic or asymptomatic contained LBVaV. This makes the Yuma area the only region of the world to date where attempts have been made to identify these viruses that LBVaV has not been found associated with big vein disease. Although MLBVV is the causative agent, it is highly unusual not to find LBVaV associated as it is transmitted by the same vector, *O. brassicae*, and routinely co-infects plants with MLBVV.

Parallel studies conducted in the Salinas Valley of California identified co-infection of LBVaV and MLBVV in 83% of plants, which was a significant association compared to random distribution of each virus (data not shown). All Salinas Valley plants with co-infection were symptomatic, but there was no statistically significant relationship between symptom severity and co-infection due to the high frequency of co-infection. Only five samples were free of both viruses, all of which were asymptomatic. Seven plants were positive for LBVaV and negative for MLBVV; all of these plants were asymptomatic. Three plants were positive for MLBVV and negative for LBVaV; all of the plants were symptomatic. The fact that both viruses are found together routinely in the Salinas Valley, yet only MLBVV is found in the Yuma area is even more surprising, since there is extensive movement of equipment back and forth between these regions, which no doubt moves the common fungal vector.

Greenhouse testing of resistant and susceptible lettuce cultivars

Inoculation with viruliferous *O. brassicae* carrying MLBVV and LBVaV resulted in significantly different percentages (χ^2 , 2df = 24.3, $p < 0.01$) of symptomatic plants among Great Lakes 65 (76% symptomatic), Margarita (31% symptomatic), and Pavane (29%) in experiment 1 (Table 1). In experiment 2, Great Lakes 65 (88% symptomatic), Pavane (38%) and *L. virosa*

accession IVT280 (0% symptomatic) also had significantly different percentages (χ^2 , 2df = 34.9, $p < 0.01$) of symptomatic plants (Table 1). Detection of MLBVV and LBVaV with RT-PCR confirmed MLBVV infection or LBVaV/MLBVV co-infection in symptomatic greenhouse-grown plants, and virus infection patterns paralleled the results from field samples (data not shown)..

Table 1. Percent symptomatic plants in inoculated greenhouse trials of Great Lakes 65, Margarita, Pavane and *L. virosa* accession IVT280.

Line	No. plants Tested	No. symptomatic plants	Percent Symptomatic
<i>Experiment 1</i>			
GL65	132	100	76
Margarita	127	40	31
Pavane	119	35	29
Total	378	175	137
χ^2 , 2 df			24.3*
<i>Experiment 2</i>			
GL65	104	91	88
IVT280	36	0	0
Pavane	106	40	38
Total	246	131	53
χ^2 , 2 df			34.9*

* p-value < 0.01

Representative samples of symptomatic lettuce collected from fields located in the Yuma production area of Arizona and the Salinas Valley of California were identified for characterization of variability among virus isolates. Samples from the Salinas Valley contained both MLBVV and LBVaV, however, samples from the Yuma area only contained MLBVV since no LBVaV could be identified from the Yuma area. RT-PCR was used to amplify a segment from the coat protein region of both MLBVV and LBVaV. Each amplicon was subsequently direct sequenced to determine variability among isolates from throughout the Yuma area, and similarity between Yuma isolates and isolates from other parts of the world. Results demonstrated that the region of the MLBVV coat protein characterized exhibited between 97 and 100% genetic conservation among Yuma isolates. This was similar to the genetic conservation among international isolates (Table 2), with the exception of an isolate from Almeria, Spain which was only 92% identical to a standard Yuma isolate (isolate Y-GV1 was chosen as a standard for international comparisons since all Yuma isolates shared similar sequence identity. Previous studies demonstrated that the Almeria isolate belongs in a separate taxonomic group of MLBVV and this is supported by the data in Table 2.

Table 2. Percent nucleotide sequence identity among amplicons of MLBVV and LBVaV coat protein coding regions from isolates representing California, Arizona, Europe and Asia¹.

International comparison of variability among MLBVV coat protein amplicons

Isolate	MUR1	ALM1	LS301-O	Japan	GER2	ITA1	Y-GV1
SAL-C6	97.5	90.9	98.5	98.5	97.3	97.8	97.6
Yuma GV1	96.8	91.9	99.3	99.3	99.0	97.1	
ITA1	95.8	91.2	96.8	96.8	96.1		
GER2	96.3	91.0	98.8	98.8			
Japan	97.6	91.7	100.0				
LS301-O	97.6	91.7					
ALM1	90.2						

International comparison of variability among LBVaV coat protein amplicons

Isolate	Japan	GAL1	ALM5	GRA1	UK2	MUR2
SAL-C6	96.8	97.7	98.1	98.7	98.1	98.1
MUR2	96.8	99.7	98.7	98.7	98.1	
UK2	96.1	97.7	98.1	98.7		
GRA1	96.8	98.4	99.4			
ALM5	96.8	98.4				
GAL1	96.4					

¹ Area of origin for MLBVV and LBVaV isolates used in comparative studies.

Isolate	Origin
LS301-O	Netherlands
ITA1	Italy
GER2	Germany
ALM1	Spain
MUR1	Spain
YGV1	Yuma, AZ, USA
MUR2	Spain
UK2	England
GRA1	Spain
ALM5	Spain
GAL1	Spain
Japan	Japan

Although no LBVaV was identified in Yuma lettuce, Salinas Valley samples did have LBVaV and allowed for comparison of western isolates of this virus with those from other parts of the world. Like the results from MLBVV, LBVaV isolates from California shared high sequence identity with each other and with isolates from other parts of the world (Table 2). This suggests it is unlikely that the reason we did not identify LBVaV in Yuma lettuce was not due to divergent sequence, but rather to lack of infection. It is possible that LBVaV is unable to survive in its vector, *O. brassicae*, during the high temperatures of the desert summer, although further studies would be necessary to confirm this hypothesis. It is unlikely that the virus was not introduced due to the extensive movement of labor and equipment between the Salinas Valley and the Yuma area.

SUMMARY

Research in big vein disease control has been limited by a lack of knowledge regarding the pathogen. Consequently, discovery of MLBVV in big vein symptomatic tissue, and its later report as the causal agent of big vein was an important breakthrough for big vein research. Determining the occurrence of MLBVV in symptomatic lettuce and understanding the genetic diversity of MLBVV isolates are important steps toward furthering big vein research in western U.S. production regions. Our research substantiates previous reports (Roggero et al. 2003; Navarro et al. 2004), showing a strong dependence between big vein symptom expression and MLBVV presence in Yuma grown lettuce. In addition our research also demonstrates that symptomatic and asymptomatic plants from both resistant and susceptible *L. sativa* cultivars can accumulate MLBVV and LBVaV (although LBVaV apparently does not accumulate in Yuma). Among wild relatives of lettuce, only accessions of *Lactuca virosa* have demonstrated a complete lack of symptom expression in inoculated trials (Bos and Huijberts, 1990). *Lactuca virosa* accession IVT280 was identified as 100% asymptomatic in the inoculated greenhouse trials reported here. Analysis by RT-PCR demonstrated no viral amplification, indicating apparent immunity in this accession. Breeding efforts using big vein immunity from IVT280 are being pursued (Hayes et al., 2004). The ability to detect MLBVV accumulation will greatly improve breeding efforts for big vein immunity derived from *L. virosa*.

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